

Nested cooperativity and salt dependence of the ATPase activity of the archaeal chaperonin Mm-cpn

Andrew R. Kusmierczyk^a, Jörg Martin^{b,*}

^aDepartment of Molecular Biology, Cell Biology and Biochemistry, Brown University, Box G-J2, Providence, RI 02912, USA

^bAbteilung Protein Evolution, Max-Planck-Institut für Entwicklungsbiologie, Spemannstrasse 35, 72076 Tübingen, Germany

Received 14 May 2003; revised 17 June 2003; accepted 18 June 2003

First published online 30 June 2003

Edited by Felix Wieland

Abstract The properties of the ATPase activity of the type II chaperonin from *Methanococcus maripaludis* (Mm-cpn) were examined. Mm-cpn can hydrolyze not only ATP, but also CTP, UTP, and GTP, albeit with different effectiveness. The ATPase activity is dependent on magnesium and potassium ions, and is effectively inhibited by sodium ions. Maximal rates of ATP hydrolysis are achieved at 600 mM potassium. Initial rates of ATP hydrolysis by Mm-cpn were determined at various ATP concentrations, revealing for the first time the presence of both positive intra-ring and negative inter-ring cooperativity in the archaeal chaperonin.

© 2003 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Chaperonin; Thermosome; ATPase; Cooperativity; Archaea

1. Introduction

Chaperonins comprise a subset of molecular chaperones which function as ATP-driven macromolecular protein folding machines [1]. Chaperonins can be classified into two types. Type I chaperonins are found in bacteria, mitochondria and chloroplasts, while type II are found in eukaryotes and archaea. All chaperonins assemble into double rings composed of ~57 kDa subunits [2,3]. In the case of bacterial GroEL, the rings consist of seven identical subunits. In contrast, the rings of the eukaryotic chaperonin CCT (containing TCP-1 (t-complex polypeptide 1)) contain eight different subunits, while the rings of archaeal chaperonins can be eight- or nine-membered, and are comprised of one to three different subunit types. The ring architecture of chaperonins allows for a large central cavity where non-native proteins may bind and undergo productive folding in a nucleotide-dependent manner [4].

To date, GroEL has been the most widely studied chaperonin, particularly in terms of elucidating the role of ATP in chaperonin action. Considerably less is known about the ATP-driven reaction cycle in archaeal thermosomes. In the absence of nucleotide, thermosomes are expected to be in a largely open conformation, with the central cavity accessible

to potential substrate proteins [5]. The binding of non-hydrolyzable nucleotide analogs is able to effect conformational changes in the thermosome [6] and bring about the protection of bound substrate from cleavage by protease [7], suggesting that nucleotide binding alone may be sufficient to close the cavity and lead to protein folding [8]. Nevertheless, the precise roles of nucleotide binding and hydrolysis in thermosome activity await further characterization.

In general, type II chaperonins have thus far proven more challenging to characterize to the same extent as GroEL. This is partly due to their subunit heterogeneity, best exemplified by the eukaryotic CCT. The simpler composition of archaeal chaperonin system could poise them to become attractive model systems for the biochemical study of the type II family were it not for the fact that many of the thermosomes that are currently under investigation are not fully functional in vitro [9–11]. This could partly be due to the fact that they are usually derived from (hyper)thermophilic sources and do not adjust well to in vitro conditions of lower temperatures. Recently, we reported that Mm-cpn, the homo-oligomeric chaperonin from the mesophilic archaeon *Methanococcus maripaludis*, could serve as a useful model system for the biochemical study of type II chaperonins because it was fully functional under ambient laboratory conditions and could support the folding of the model substrate rhodanese [8]. In the present study, we examine the properties of the ATPase activity of Mm-cpn and present the first evidence for the presence of both positive and negative cooperativity in an archaeal chaperonin.

2. Materials and methods

2.1. Protein purification

Mm-cpn and GroEL were purified as described [4,8,12].

2.2. ATPase activity of Mm-cpn

Nucleotide hydrolysis activity was determined as described [13] and liberated P_i was determined by a Malachite Green assay [14]. Briefly, ATP (1.5 mM) was added to samples containing Mm-cpn and hydrolysis was allowed to proceed for various times. The reaction was stopped with 10 mM EDTA, followed by colorimetric detection. To determine the cation-dependence of Mm-cpn ATPase activity, Mm-cpn (110 nM final oligomer) or GroEL (50 nM final oligomer) was added to buffer A (30 mM Tris, pH 7.5, 5 mM $MgCl_2$) at 37°C, and supplemented with various salts as indicated in the figure legends. For determination of pH dependence, 30 mM bisTris-HCl and 30 mM Tris-HCl systems were used at the indicated pH values, each buffer being supplemented with 5 mM $MgCl_2$ and 100 mM KCl.

2.3. Determination of cooperativity

Initial rates of ATP hydrolysis were measured by determining lib-

*Corresponding author. Fax: (49)-7071-601 349.

E-mail addresses: andrew_kusmierczyk@brown.edu (A.R. Kusmierczyk), joerg.martin@tuebingen.mpg.de (J. Martin).

Abbreviations: CCT, chaperonin containing TCP-1 (t-complex polypeptide 1); Mm-cpn, *Methanococcus maripaludis* chaperonin

erated Pi using radiolabeled [γ - 32 P]ATP, with sample processing as described [15], or by an improved colorimetric assay according to Geladopoulos et al. [16]. Reaction rates were measured in buffer A plus 100 mM KCl. Aliquots were withdrawn at 2, 4, 6, 8, 10 min (or at 1.5, 3, 4.5, 6, 7 min for [ATP] < 20 μ M), to confirm that hydrolysis remained linear with time. Curve fitting was performed using Kaleida-Graph 3.5 (Synergy Software Inc.) and, where indicated, the data were fit to the following Hill-type equation [17]:

$$V = (V_{\max 1} + V_{\max 2}(L/K_2)^m)/(1 + (K_1/L)^n + (L/K_2)^m) \quad (1)$$

where V is the observed rate of ATP hydrolysis, L is the ATP concentration, $V_{\max 1}$ and $V_{\max 2}$ are the maximum rates of hydrolysis by one and both rings of Mm-cpn, K_1 and K_2 are the apparent binding constants of ATP by the first and second rings, and n and m are the Hill coefficients for the first and second allosteric transitions.

3. Results and discussion

3.1. Properties of the Mm-cpn ATPase activity

ATPase activity is essential to chaperonin function in vivo and in vitro [1,4,12,18]. We incubated Mm-cpn at 37°C in the presence of 1.5 mM of various nucleotide triphosphates for up to 20 min and quantitated the liberated inorganic phosphate. Over the course of the assay, the amount of liberated inorganic phosphate increased linearly with time (not shown), and we found that in addition to ATP, Mm-cpn could hydrolyze CTP to a similar extent, while UTP and GTP were hydrolyzed less efficiently (Fig. 1). These results are similar to those reported for the chaperonin from *Methanococcus thermolithotrophicus* which could promote recovery of guanidine-denatured citrate synthase in the presence of ATP, CTP, and UTP, but not GTP [19]. The ATPase activity of Mm-cpn was dependent on the presence of K^+ or NH_4^+ ions (Fig. 2), with K^+ being more effective at supporting hydrolysis. There was no hydrolysis in the presence of Na^+ ions. Sulfate appeared to have a mildly inhibitory effect in the context of K^+ ions. Stimulation of ATPase activity by K^+ has been observed for other chaperonins as well. The ATPase activity of GroEL is strictly dependent on K^+ ions [20], whereas the stimulatory gains for CCT in the presence of K^+ are more modest [21]. Among the archaea, potassium strongly stimulates the ATPase activity of the thermosome from *Sulfolobus solfataricus* [22] and has a mild stimulatory effect on the thermosome from *Thermoplasma acidophilum* [23]. In contrast, the thermosome of *Methanopyrus kandleri* is strictly dependent on high concentrations of ammonium ions [10]. Magnesium is in-

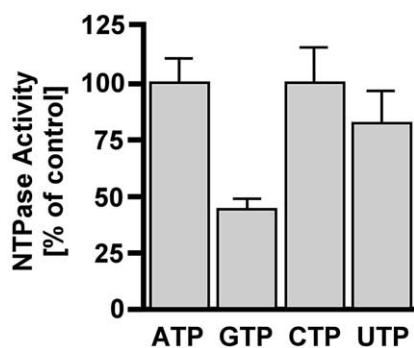


Fig. 1. Ability of Mm-cpn to hydrolyze various nucleotide triphosphates. Mm-cpn was incubated in buffer A with 100 mM KCl at 37°C and supplemented with 1.5 mM of the indicated nucleotides to initiate hydrolysis. Activity obtained with ATP is set to 100%.

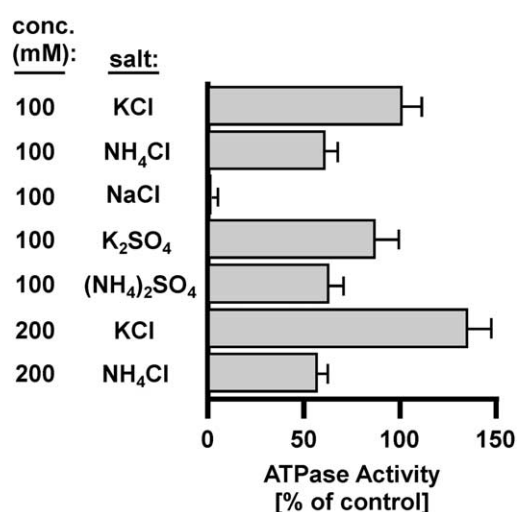


Fig. 2. Salt dependence of Mm-cpn ATPase activity. Mm-cpn was incubated in buffer A with different salts at the indicated concentrations. Initial rates of ATP hydrolysis were determined as described in Section 2. Activity at 100 mM KCl is set to 100%.

involved in coordination of the nucleotide [24] and, as with all chaperonins, the ATPase activity of Mm-cpn is dependent on the presence of Mg^{2+} . Consistent with this, calcium ions could not support ATP hydrolysis by Mm-cpn (not shown). We found no difference in ATP hydrolysis when the Mg^{2+} concentration was varied from 1 to 50 mM (not shown).

The ATPase activity of Mm-cpn was found to vary strongly with the concentration of potassium ions (Fig. 3A), whereas no such effect was seen with ammonium cations (see also Fig. 2). No significant ATPase activity was detected when the concentration of K^+ was below 10 mM, and activity was maximal at 600 mM K^+ . Moreover, in addition to not supporting ATP hydrolysis (Fig. 2), Na^+ ions were found to significantly inhibit the K^+ -dependent ATP hydrolysis by Mm-cpn (Fig. 3B). With ATP hydrolysis in buffer containing 100 mM KCl at 37°C set as a control, the addition of an equimolar amount of NaCl resulted in a 93% inhibition of the ATPase activity of Mm-cpn compared to only 27% for GroEL (Fig. 3B). A recent high resolution crystal structure of GroEL suggests the involvement of potassium in the positioning of the γ -phosphate of ATP for hydrolysis [24]. The greater susceptibility of archaeal chaperonins to inhibition by Na^+ could be the result of a lower relative affinity for K^+ at the nucleotide binding site, thereby allowing for more efficient competition by the former. While inhibition of the ATPase activity by sodium ions has also been observed in the chaperonin from the archaeon *Haloferax volcanii* [11], the absolute salt concentrations used in that study were in the molar range, reflecting the halophilic nature of the *Haloferax* protein. As a result, the effects of sodium ions on halophilic chaperonins could additionally be due to ionic surface binding effects. Methanogens have been shown to accumulate high levels of intracellular potassium, which can range from approximately 150 mM for mesophilic species, such as *Methanospirillum hungatei*, to over 1.2 M for certain thermophilic species, such as *Methanobrevibacter arboriphilus* [25,26]. However, sodium levels in methanogenic archaea are maintained at levels that are significantly lower than potassium levels and, in certain methanogenic archaea, are kept even lower than in the outside me-

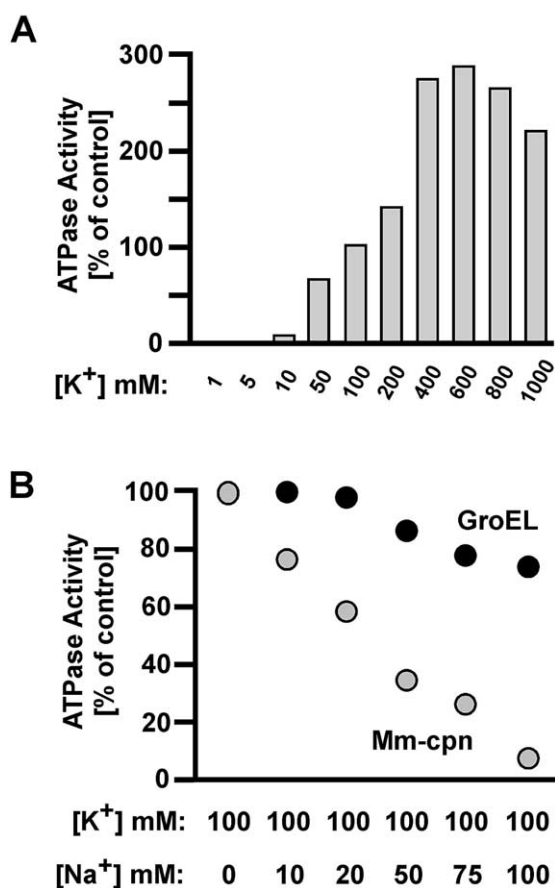


Fig. 3. Opposing effects of potassium and sodium ions on chaperonin ATPase activities. A: Mm-cpn was incubated in buffer A with increasing concentrations of KCl and initial rates of ATP hydrolysis were determined. Activity at 100 mM KCl is set to 100%. B: Mm-cpn and GroEL were incubated in buffer A with 100 mM KCl and increasing concentrations of NaCl. ATPase activities were determined as described in Section 2. Activity in the absence of NaCl is set to 100%.

dium [25]. Thus, inhibition by sodium is likely not to be problematic for Mm-cpn in vivo.

To determine the pH dependence of Mm-cpn ATPase activity, we employed an overlapping buffer system to cover a pH range from 5.5 to 9.0 (Fig. 4). The ATPase activity of Mm-cpn approached background hydrolysis levels below pH 6.0, and was maximal at pH 8.0. *M. maripaludis* can be grown in culture between a pH of 6.4 and 7.8, with maximal growth occurring between pH 6.8 and 7.2 [27].

3.2. Nested cooperativity in ATP hydrolysis

Measuring the ATPase activity as a function of ATP concentration can reveal important information about intra- and inter-ring cooperativity. The curve profile in Fig. 5A shows such a data set for Mm-cpn obtained under experimental conditions where Mm-cpn has been shown to be fully functional in mediating protein folding of substrate proteins [8]. The profile is reminiscent of what has been described for eukaryotic CCT, with two distinct transitions representing the loading of the first and second rings with ATP [17]. The data in the low concentration range (<150 μ M; Fig. 5B) were fitted to the Hill equation (Fig. 5B, inset) and the initial parameters were used to constrain Eq. 1 in order to obtain a

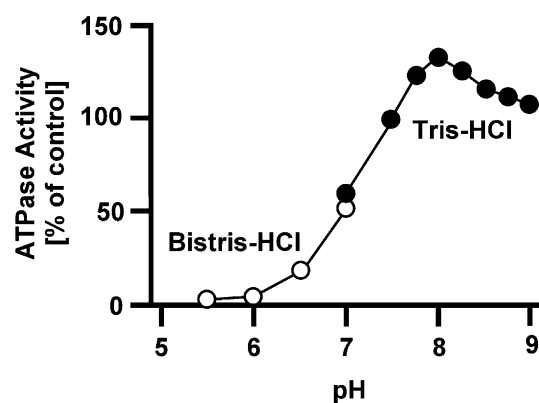


Fig. 4. pH dependence of Mm-cpn ATPase activity. Initial rates of nucleotide hydrolysis by Mm-cpn were measured after incubation of samples in bisTris-HCl or Tris-HCl buffers at the indicated pH values. Activity at pH 7.5 is set to 100%.

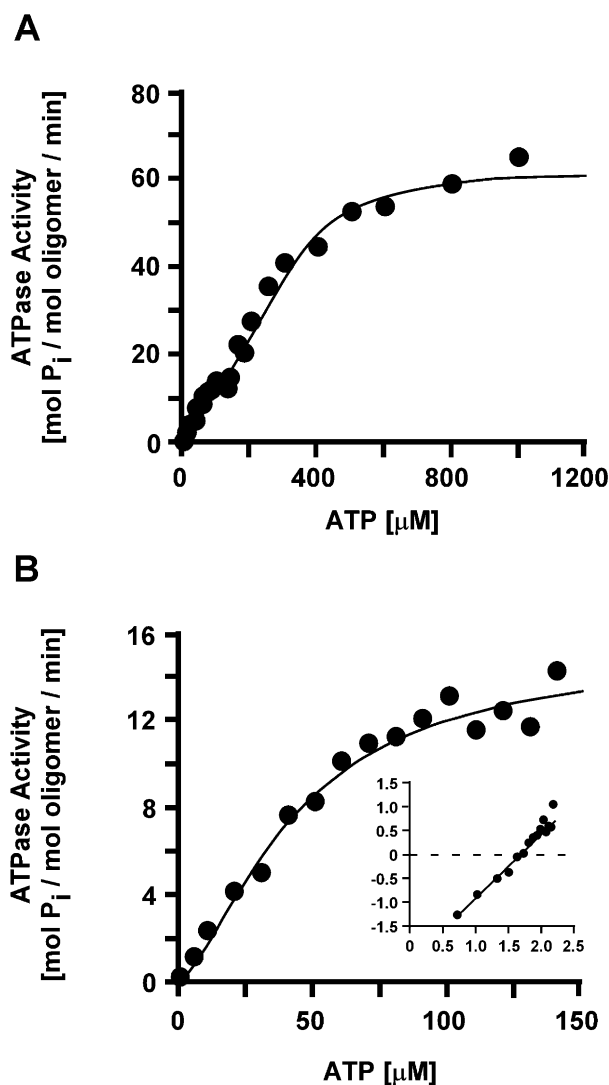


Fig. 5. Nested cooperativity in ATP hydrolysis of Mm-cpn. A: Observed velocities across the full range of ATP concentrations used. Data were fit to Eq. 1. B: Close-up view of observed velocities across the low range of ATP concentrations. Data were fit to the Hill equation. Inset: Hill plot of data in B. Y-axis: $\log(V/(V_{\max} - V))$, X-axis: $\log[L]$.

good fit for the entire data set. We obtained the following parameters for the first ring: an apparent binding constant, K_1 , of $43 (\pm 8) \mu\text{M}$; $V_{\max 1}$ of $15.2 (\pm 1.6) \text{ ATP min}^{-1}$; and a Hill coefficient of $1.47 (\pm 0.26)$. For the second ring, the apparent binding constant, K_2 , was found to be $296 (\pm 17) \mu\text{M}$, while the Hill coefficient was $2.97 (\pm 0.34)$. The rate of hydrolysis, $V_{\max 2}$, for both rings was determined to be $59.4 (\pm 2.1) \text{ ATP min}^{-1}$, a steady-state rate that is comparable to that of GroEL [28]. Since the Hill coefficients for each ring in Mm-cpn are > 1 , this suggests the presence of positive intra-ring cooperativity. Our findings contrast with an earlier attempt at determining the cooperative properties of an archaeal chaperonin, from *T. acidophilum*, which suggested a slight negative cooperativity [23]. However, the low intrinsic ATPase rate of that particular chaperonin precluded measurements at very low ATP concentrations [23]. In contrast, at 37°C , the ATPase rate of Mm-cpn is sufficiently high to use low enough protein concentrations ($< 200 \text{ nM}$ oligomer) to obtain data even at low ATP concentrations. The Mm-cpn data presented here indicate that archaeal chaperonins exhibit positive intra-ring cooperativity, as is the case for bacterial GroEL and eukaryotic CCT [17,28]. Moreover, since $K_2 > K_1$ and since $m > n$ for Mm-cpn, this is consistent with the presence of negative cooperativity between rings, as was argued in the case of eukaryotic CCT [17]. It should be noted that the difference between the apparent affinity constants for ATP binding to each ring (K_1 versus K_2), as well as the difference between the Hill coefficients for the two transitions (n versus m), is smaller in Mm-cpn than in CCT, despite similar experimental conditions (100 mM K^+ and 37°C versus 50 mM K^+ and 25°C in the CCT study). The aforementioned differences in affinity constants and Hill coefficients could be due to a lesser extent of negative cooperativity in Mm-cpn than in CCT, and may explain why V_{\max} of both rings is nearly four times greater than V_{\max} of a single ring in the archaeal chaperonin, compared to a factor of two in the eukaryotic one [17]. Lower negative cooperativity in Mm-cpn compared to CCT could be due to a difference in the nucleotide binding mode between subunits in the hetero-oligomeric CCT rings and those in the homo-oligomeric Mm-cpn rings. In the hetero-oligomeric CCT, genetic evidence supports a sequential, Koshland–Némethy–Filmer-type, mechanism of nucleotide association in a ring, consistent with the proposed order of the eight different subunits within a ring [28–30]. Consequently, the second ring might also have to be loaded in a particular order. In contrast, it is difficult to envision how sequential cooperativity would work in a homo-oligomeric complex such as Mm-cpn. The equivalence of subunits within an Mm-cpn ring may impose no hierarchical requirement to initiate binding at a particular subunit in a given ring, as was suggested to occur in CCT [30]. Instead, Mm-cpn may display a concerted, Monod–Wyman–Changeux-type, mode of nucleotide association within a ring, akin to what has been proposed for the homo-oligomeric GroEL [31,32]. GroEL also displays negative cooperativity when it comes to inter-ring communication [32]. However, unlike in GroEL, where a major consequence is a decrease in the rate of ATP hydrolysis in both rings, the V_{\max} for both rings of Mm-cpn and CCT is larger than V_{\max} for one ring. This suggests a different mode of inter-ring communication in type II chaperonins, which could be the result of a different positioning of the two chaperonin rings relative to each other, namely stag-

gered in type I versus in-register in type II chaperonins [2,3]. Nevertheless, the above data for Mm-cpn confirm for the first time the universality of dual cooperativity (positive intra-ring and negative inter-ring) among chaperonins from all three domains of life.

Acknowledgements: We thank Astrid Ursinus for excellent technical assistance.

References

- [1] Gutsche, I., Essen, L.-O. and Baumeister, W. (1999) *J. Mol. Biol.* 293, 295–312.
- [2] Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D.C., Joachim, A., Horwich, A.L. and Sigler, P.B. (1994) *Nature* 371, 578–586.
- [3] Ditzel, L., Löwe, J., Stock, D., Stetter, K.-O., Huber, H., Huber, R. and Steinbacher, S. (1998) *Cell* 93, 125–138.
- [4] Langer, T., Pfeifer, G., Martin, J., Baumeister, W. and Hartl, F.U. (1992) *EMBO J.* 11, 4757–4765.
- [5] Nitsch, M., Walz, J., Typke, D., Klumpp, M., Essen, L.O. and Baumeister, W. (1998) *Nat. Struct. Biol.* 5, 855–857.
- [6] Gutsche, I., Holzinger, J., Rauh, N., Baumeister, W. and May, R.P. (2001) *J. Struct. Biol.* 135, 139–146.
- [7] Yoshida, T., Kawaguchi, R., Taguchi, H., Yoshida, M., Yasunaga, T., Wakabayashi, T., Yohda, M. and Maruyama, T. (2002) *J. Mol. Biol.* 315, 73–85.
- [8] Kusmierczyk, A.R. and Martin, J. (2003) *Biochem. J.* 371, 669–673.
- [9] Minuth, T., Frey, G., Lindner, P., Rachel, R., Stetter, K.O. and Jaenicke, R. (1998) *Eur. J. Biochem.* 258, 837–845.
- [10] Andrä, S., Frey, G., Jaenicke, R. and Stetter, K.O. (1998) *Eur. J. Biochem.* 255, 93–99.
- [11] Large, A.T., Kovacs, E. and Lund, P.A. (2002) *FEBS Lett.* 532, 309–312.
- [12] Martin, J., Mayhew, M., Langer, T. and Hartl, F.U. (1993) *Nature* 366, 228–233.
- [13] Kusmierczyk, A.R. and Martin, J. (2000) *J. Biol. Chem.* 275, 33504–33511.
- [14] Lanzetta, P.A., Alvarez, L.J., Reinach, P.S. and Candia, O.A. (1979) *Anal. Biochem.* 100, 95–97.
- [15] Stark, M. and Cundliffe, E. (1979) *J. Mol. Biol.* 134, 767–779.
- [16] Geladopoulos, T.P., Sotiropoulos, T.G. and Evangelopoulos, A.E. (1991) *Anal. Biochem.* 192, 112–116.
- [17] Kafri, G., Willison, K.R. and Horovitz, A. (2001) *Protein Sci.* 10, 445–449.
- [18] Lin, P., Cardillo, T.S., Richard, L.M., Segel, G.B. and Sherman, F. (1997) *Genetics* 147, 1609–1633.
- [19] Furutani, M., Iida, T., Yoshida, T. and Maruyama, T. (1998) *J. Biol. Chem.* 273, 28399–28407.
- [20] Viitanen, P.V., Lubben, T.H., Reed, J., Goloubinoff, P., O'Keefe, D.P. and Lorimer, G.H. (1990) *Biochemistry* 29, 5665–5671.
- [21] Roobol, A., Grantham, J., Whitaker, H.C. and Carden, M.J. (1999) *J. Biol. Chem.* 274, 19220–19227.
- [22] Guagliardi, A., Cerchia, L., Bartolucci, S. and Rossi, M. (1994) *Protein Sci.* 3, 1436–1443.
- [23] Gutsche, I., Mihalache, O. and Baumeister, W. (2000) *J. Mol. Biol.* 300, 187–196.
- [24] Wang, J. and Boisvert, D.C. (2003) *J. Mol. Biol.* 327, 843–855.
- [25] Sprott, G.D. and Jarrell, K.F. (1981) *Can. J. Microbiol.* 27, 444–451.
- [26] Sprott, G.D. and Jarrell, K.F. (1984) *Can. J. Microbiol.* 30, 663–668.
- [27] Jones, W.J., Paynter, M.J.B. and Gupta, R. (1983) *Arch. Microbiol.* 135, 91–97.
- [28] Koshland, D.E., Némethy, G. and Filmer, D. (1966) *Biochemistry* 5, 365–385.
- [29] Liou, A.K. and Willison, K.R. (1997) *EMBO J.* 16, 4311–4316.
- [30] Lin, P. and Sherman, F. (1997) *Proc. Natl. Acad. Sci. USA* 94, 10780–10785.
- [31] Monod, J., Wyman, J. and Changeux, J.P. (1965) *J. Mol. Biol.* 12, 88–118.
- [32] Yifrach, O. and Horovitz, A. (1995) *Biochemistry* 34, 5303–5308.